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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Initial characterization of the ability of the human Reaper homolog (hRpr) to initiate apoptosis in human cell lines has proven to be difficult. Possible explanations for the difficulty in working with hRpr include low expression levels and the potential requirement for a second protein to cooperate or synergize with hRpr in order to initiate apoptosis. However, many of the goals set out in the statement of work have been achieved, including the construction of hRpr expression vectors and successful transfection of hRpr into human cell lines. Additionally, numerous apoptosis assays have been perfected, setting the stage for successful analysis once the correct conditions are for hRpr expression and activity are worked out. Furthermore, research that deviated from the original statement of work has led to a publication and strengthened my ability to characterize hRpr. For example, studies of Reaper itself have revealed that the interaction between Reaper and IAPs results in the ubiquitination and degradation of both proteins. This proteolytic regulation of Reaper may also explain the difficulty expressing hRpr. Additionally, we have found that Reaper inhibits translation, which opens up another exciting avenue for characterizing the activity of hRpr.

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Annual summary report

PI: Christopher Holley

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Introduction

One of the processes at the heart of tumor development is the ability of a cell to evade apoptosis. Although the core execution machinery of the apoptotic program is well described, much less is known about cytoplasmic proteins that initiate the 'intrinsic' apoptotic signaling cascade. Of these, one of the best studied is the *Drosophila* protein Reaper. This pro-apoptotic protein is induced by normal hormone signaling during development of the fly embryo, and it can also be induced by ionizing radiation in a p53-dependent manner. While Reaper is a key regulator of apoptosis in the fly, there are no published reports of a human homolog. However, work in our laboratory and others has shown that the Reaper protein retains its ability to induce apoptosis in the context of vertebrate cells. The potential for a human Reaper homolog with a role in p53-mediated apoptosis following ionizing radiation led us identify a putative homolog, located on chromosome 16 of the human genome. The essence of the proposal funded by the award noted above was to characterize this human Reaper homolog (hRpr).

Body

Task 1 of the research proposal was to investigate the biological significance of hRpr in radiation induced apoptosis. The first step in this process was to develop a mammalian expression construct in order to transfect human cells with hRpr (Task 1A1).. To date, untagged hRpr has been successfully cloned into the expression vectors pcDNA3 and pEBB. Although hRpr is expressed only at low levels following transfection, I have observed at least some expression in several human cell lines (HeLa, MCF7, and 293T). After validating expression, I attempted a variety of assays to measure hRpr's ability to initiate apoptosis. Approaches I have used include direct

measurement of caspase activity (using a colorimetric substrate), TUNEL staining, fluorescently labeled caspase substrates (Phi-Phi-Lux), annexin V-PI staining, and most recently, co-transfection with GFP followed by FACS analysis, as outlined in the statement of work (Tasks 1A2 and 1A3). Unfortunately, the results have been quite disappointing. By itself, hRpr appears to be a weak inducer of apoptosis, if at all. The weak phenotype of hRpr in combination with difficulties obtaining transfectable, radiosensitive cell lines (see below) has prevented successful assessment of any synergy between hRpr and radiation (Task 1A4).

One reason for these results may be that hRpr is only weakly expressed (as detected by immunoblotting). This may be due to an inherent instability and rapid turnover of the hRpr protein. Corroborating evidence for this hypothesis has come from work I have done, outside of the scope of my original proposal, on the stability of Reaper itself. Reaper is also difficult to express, but it is somewhat easier to detect than hRpr, so I examined the issue of stability using Reaper. It has been known for some time that the N-terminus of Reaper interacts with IAPs (inhibitors of apoptosis), and recently, certain IAPs with RING finger motifs have been shown to have intrinsic E3-ligase activity. Historically, the interaction between Reaper and the IAPs was thought to lead to simple competitive inhibition of the IAP, as Reaper blocked the ability of IAPs to inhibit caspases. However, we have shown that the interaction of Reaper with the IAPs also results in auto-ubiquitination of the IAP, leading to lowered IAP levels and a consequent lowering of the threshold for apoptosis (Holley et al. 2002). Conversely, we have observed that the IAP also ubiquitinates Reaper, catalyzing its destruction by the ubiquitin-proteasome pathway. Mutations of Reaper that prevent association with the IAP (Reaper N-terminal truncation) or block ubiquitination (lysine mutants) greatly stabilize the Reaper protein by preventing its destruction (Olson et al., manuscript in preparation). Thus, Reaper and IAPs seem locked in a struggle to destroy each other, and the winner of this destructive race seems to be determined by relative expression levels. Although I have not yet formally tested the stability of hRpr with respect to ubiquitin-mediated proteolysis, this will be an interesting avenue to pursue. It may be that the context of normal hRpr expression (such as in response to ionizing radiation) allows for

stabilization of the hRpr protein, while it is destroyed too rapidly to induce apoptosis in the context of transfected but otherwise untreated cells.

Additionally, recent reports of proteins that share limited functional homology to Reaper may shed light on the weak phenotype of hRpr expression alone. Like Reaper, these proteins (Smac/DIABLO, Omi/HtrA2, and others) interact with and antagonize the IAPs. However, like hRpr, these proteins are insufficient to initiate the apoptotic program alone. Additionally, these proteins have none of the other characteristics associated with Reaper, such as transcriptional regulation (radiation- and hormone-inducibility) and the ability to release mitochondrial cytochrome c. The hRpr protein, however, does have these characteristics – but hRpr does not seem to interact directly with the IAPs. It therefore seems possible that the functions of IAP inhibition and cytochrome c release are combined in one fly protein (Reaper) that can alone induce apoptosis, but are associated with two or more proteins in mammals (hRpr and Smac/DIABLO) which must be concurrently active to initiate the apoptotic program. I have tested this hypothesis by co-transfection of hRpr and DIABLO, and the initial results seem promising. That is, transfection of hRpr or DIABLO alone has no effect on cultured cells, but co-transfection of the two results is apoptosis. This is a deviation from the original statement of work, but it seemed warranted in light of the disappointing results obtained with hRpr alone and in light of the characterization of the Smac/DIABLO proteins.

Another important aspect of the proposed work was to investigate the relationship between hRpr and radiation-induced apoptosis. Although I have demonstrated that hRpr is induced by radiation in the human ML-1 cell line, I have found that these cells cannot be transfected in a way that allows for quantitation of apoptosis (electroporation results in too many dead cells). I have thus looked for other radiosensitive cell lines. Unfortunately, it has been extremely difficult to find a transfectable cell line that dies by apoptosis in response to ionizing radiation. For the most part, available cell lines are mutated for p53 and are not sensitive to radiation-induced damage. Of those cell lines that have maintained functional p53, nearly all of them exhibit a non-apoptotic response to ionizing radiation (i.e., growth arrest). While I am continuing to search for cell lines which are (a) transfectable and (b) die by apoptosis (with induction of hRpr) in response

to ionizing radiation, I have been as yet unable to achieve any of the goals regarding the effect of antisense hRpr on radiation-induced apoptosis (Task 1B). However, I am hopeful that these goals will be met once a suitable cellular context is found.

Despite the initial difficulties encountered working with the hRpr protein, I remain convinced that it is a *bona fide* Reaper homolog. I have further substantiated the relationship between the two proteins using computational algorithms to predict the secondary structure of each protein. The result of this analysis shows that both Reaper and hRpr are strongly predicted to exhibit extensive alpha-helical structure.

Looking forward, months 12-36 of the training grant will include investigation of translational control with respect to hRpr expression. To that end, I have recently obtained bicistronic reporter constructs in order to explicitly test the function of the putative IRES within the intra-ORF region of the hRpr mRNA (Task 2B). Also, our laboratory has recently shown that the Reapar protein is capable of inhibiting translation (Holley et al. 2002). While the mechanism of this effect is not yet clear, inhibition of cap-dependent translation would fit with IRES-mediated translation of hRpr (and potentially Reaper itself; although the Reaper mRNA is not bicistronic, it may still contain an IRES). In order to examine the ability of Rpr to inhibit translation, we have developed an assay to measure the rate of protein translation in *Xenopus* oocytes that have been injected with S-35 labeled amino acids. Co-injection of Reaper mRNA inhibits the incorporation of S-35 into TCA-precipitable counts, and I plan to perform this assay using hRpr mRNA. If hRpr can also inhibit translation, it will be very interesting to see whether the putative hRpr IRES can continue to support hRpr synthesis.

Key accomplishments

- development of hrpr mammalian expression constructs
- expression of hrpr by transfection of human cells in culture
- development of apoptosis assays, including use of GFP as a marker
- analysis of hrpr predicted secondary structure
- acquisition of bicistronic reporter constructs for analysis of the hrpr IRES
- recognition of hRpr instability as a potential regulator of hRpr activity
- identification of translational inhibition as potential activity of hRpr

Reportable outcomes

Manuscripts

Holley CL, Olson MR, Colon-Ramos DC, and Kornbluth S. (2002) Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat. Cell Biol.* 4:439.

Olson MR, Holley CL, Kornbluth S. (in preparation) IAP proteins stimulate Reaper degradation via the ubiquitin-proteasome pathway.

Abstracts

Holley CL, Olson MR, Colon-Ramos DC, and Kornbluth S. Mechanisms of Reaper-induced apoptosis. Gordon Research Conference on Cell Death. Colby College, Waterville, ME. June 16-21, 2002

Holley CL and Kornbluth S. A radiation-inducible human apoptotic regulator with homology to *Drosophila* Reaper. Cold Spring Harbor Cell Death Meeting. November 9-13, 2001.

Presentations

Holley CL, Olson MR, Colon-Ramos DC, and Kornbluth S. Mechanisms of Reaper-induced apoptosis. Duke University Medical Scientist Training Program Research Symposium. Durham, NC. March 3, 2002.

Holley CL and Kornbluth S. Regulation of apoptosis by a human reaper homolog. Duke University Biological Science Graduate Student Symposium. Durham, NC. October 26, 2001.

Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition

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focus on *apoptosis*

Inhibitors of apoptosis (IAPs) inhibit caspases, thereby preventing proteolysis of apoptotic substrates. IAPs occlude the active sites of caspases to which they are bound[1–3] and can function as ubiquitin ligases. IAPs are also reported to ubiquitinate themselves and caspases[4,5]. Several proteins induce apoptosis, at least in part, by binding and inhibiting IAPs. Among these are the *Drosophila melanogaster* proteins Reaper (Rpr), Grim, and HID, and the mammalian proteins Smac/Diablo and Omi/HtrA2, all of which share a conserved amino-terminal IAP-binding motif[6–14]. We report here that Rpr not only inhibits IAP function, but also greatly decreases IAP abundance. This decrease in IAP levels results from a combination of increased IAP degradation and a previously unrecognized ability of Rpr to repress total protein translation. Rpr-stimulated IAP degradation required both IAP ubiquitin ligase activity and an unblocked Rpr N terminus. In contrast, Rpr lacking a free N terminus still inhibited protein translation. As the abundance of short-lived proteins are severely affected after translational inhibition, the coordinated dampening of protein synthesis and the ubiquitin-mediated destruction of IAPs can effectively reduce IAP levels to lower the threshold for apoptosis.

To evaluate the effects of Rpr on the function of IAPs, we cotransfected human 293T cells with untagged Rpr and human members of the IAP family: XIAP and cIAP1. In the presence of Rpr, IAP steady-state levels were much lower than in the presence of vector alone, suggesting that Rpr was preventing XIAP and cIAP1 protein accumulation (Fig. 1a). Similar results were obtained in fly embryos, where overexpression of Rpr resulted in barely detectable levels of DIAP1 (B. Hay, personal communication). Note that 'laddered' forms of XIAP, indicative of ubiquitination, were recognized by anti-ubiquitin antibody (Fig. 1b), consistent with previous reports of IAP auto-ubiquitination[4,5].

We therefore hypothesized that Rpr might stimulate IAP ubiquitination and degradation. To determine whether Rpr affects IAP half-life, we performed pulse-chase analyses on cells cotransfected with XIAP and either Rpr or vector alone. Cotransfection with Rpr significantly affected XIAP stability (Fig. 1c; see also Fig. 3b). Moreover, Rpr greatly increased the appearance of laddered XIAP species. This change in IAP stability was not a consequence of Rpr-induced apoptosis, as the pulse-chase experiments were performed in the presence of the broad-spectrum caspase inhibitor zVAD-fmk.

To address the effects of Rpr on IAP stability in an alternative system, we examined the half-lives of radiolabelled human IAPs added to whole-cell lysates prepared from *Xenopus laevis* eggs, which reconstitute both apoptotic signalling and ubiquitin-dependent proteolysis[15,16]. Radiolabelled, *in vitro* -translated cIAP1 and XIAP proteins were added to egg extracts supplemented with vehicle or with full-length, untagged Rpr, prepared by complete *de novo* peptide synthesis[17]. As in cultured cells, Rpr addition to egg extracts significantly destabilized both cIAP1 and XIAP (Fig. 1d). Similar results were obtained with a fly IAP, DIAP1 (Fig. 4c).

To extend these findings, we isolated a *Xenopus* XIAP homologue, XLX. Domain analysis of XLX revealed two complete and one partial N-terminal baculovirus inhibitory repeat (BIR) domain, and a carboxy-terminal RING domain (Fig. 2a). In common with XIAP, XLX lacks the caspase activation recruitment domain (CARD) found in cIAP1 and cIAP2 (ref. 18). Despite truncation of BIR domain 1 in our clone, we believe XLX to be full-length, as the cDNA isolated contains three in-frame stop codons within the 5′-untranslated region (UTR) preceding the start methionine.

Because IAPs can be caspase substrates (Fig. 2b), the disappearance of IAPs in our extracts might have been caused, at least in part, by caspase-mediated cleavage[19,20]. In fact, glutathione *S*-transferase (GST)–Rpr induces mitochondrial cytochrome *c* release, thereby activating caspases in the extract[21]. Similarly, addition of Rpr peptide to crude *Xenopus* egg extracts triggered caspase activation, although at the concentration used in our IAP experiments (100 ng μl^{-1}), caspase activation was relatively delayed (Fig. 2c). However, as reported for GST–Rpr[21], the Rpr peptide could not induce caspase activation in egg cytosol lacking mitochondria (Fig. 2d; note caspase activation by cytochrome *c* addition to the same extract). Nevertheless, in these cytosolic extracts, the Rpr peptide significantly accelerated the destruction of XLX (Fig. 2e,f). XLX cleavage fragments were absent in these extracts (Fig. 2e, arrowheads) and in crude extracts incubated with zVAD-fmk (data not shown). Therefore, although caspases can cleave XLX, they are not essential for Rpr-accelerated IAP destruction. In contrast to the Rpr peptide, GST–Rpr (whose IAP-binding N terminus is shielded by its GST tag), failed to accelerate XLX destruction (Fig. 2e,f). These data suggest that Rpr-stimulated degradation of IAPs can occur independently of caspase activation, and that this effect requires the N terminus of Rpr to be unblocked. Consistent with the hypothesis that Rpr requires a free N terminus to promote IAP degradation, GST–Rpr and XIAP did not co-precipitate (Fig. 3a, right). In addition, an untagged Rpr lacking amino acids 1–15 (Rpr^{16-65}) could not promote IAP degradation, further demonstrating that the extreme N terminus of Rpr is required to shorten IAP half-life (Fig. 3b).

To determine whether IAP ubiquitin ligase activity was required for Rpr-induced IAP degradation, we cotransfected cells with Rpr and a catalytically inactive XIAP point mutant of $XIAP^{H467A}$ (ref. 4;

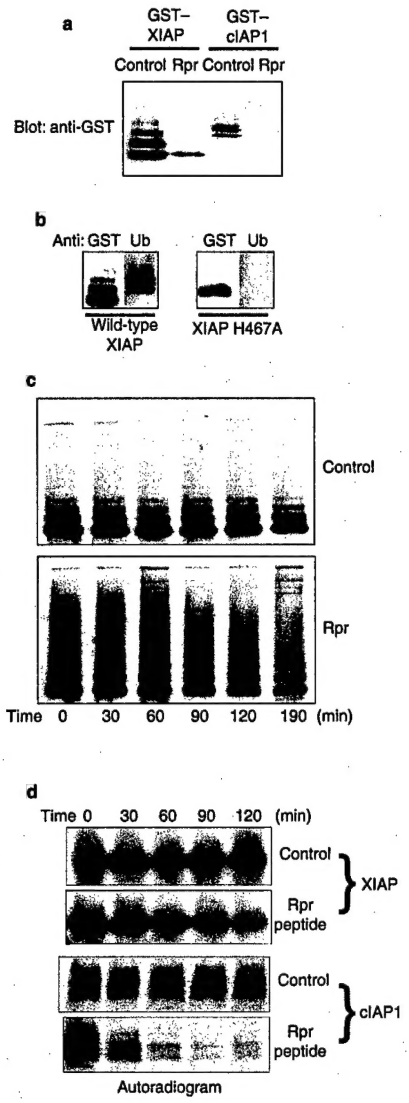

Figure 1 **Rpr stimulates IAP auto-ubiquitination and destruction. a**, HEK 293T cells were transfected with GST–XIAP or GST–cIAP1 and either Rpr or vector control, in the presence of zVAD-fmk. Following precipitation with glutathione–Sepharose, GST fusion protein levels were analysed by immunoblotting with an anti-GST polyclonal antibody. **b**, HEK 293T cells were transfected with either wild-type ubiquitin ligase or an XIAP^{H467A} mutant and precipitated with glutathione–Sepharose. Precipitates were immunoblotted with an antibody against GST or ubiquitin. **c**, HEK 293T cells were transfected with GST–XIAP and either Rpr or vector control. Cells were then radiolabelled in a pulse-chase experiment. The resulting radiolabelled GST fusion proteins were analysed by autoradiography. **d**, Radiolabelled IAPs were incubated in crude *Xenopus* egg extracts, in the presence of either Rpr peptide or vehicle control. The resulting radiolabelled protein levels were analysed by autoradiography. Equal loading was verified by Coomassie blue staining of gels (data not shown).

see also Fig. 1b). Although Rpr bound to XIAP^{H467A} (Fig. 4a), it failed to accelerate destruction of the mutant in a pulse-chase experiment (Fig. 4b). Rpr had similar effects on its *Drosophila* target, DIAP1. Again, destabilization was dependent on an intact RING domain, as the DIAP1 ubiquitin ligase mutant DIAP1^{C412Y} was not significantly destabilized (Fig. 4c). Collectively, these data demonstrate that Rpr-stimulated IAP degradation requires that the IAP be functional as a ubiquitin ligase.

Although untagged, full-length Rpr substantially destabilized all of the wild-type IAP proteins tested, we were surprised to find that Rpr also moderately decreased steady-state levels of an unrelated protein after cotransfection of human cells (Fig. 5a, GST). Additionally, overexpression of Rpr in flies lowers the levels of a DIAP1 ubiquitin ligase mutant, implying that Rpr has effects *in vivo* that are independent of its effects on IAP half-life (B. Hay, personal communication). This prompted us to examine whether IAP abundance might also be affected at the level of protein production. Indeed, when we programmed reticulocyte lysates with XIAP or XLX, IAP levels were profoundly decreased by GST–Rpr and essentially eliminated by the Rpr peptide (Fig. 5b). GST, or other unrelated proteins, had no effect (Fig. 5b and data not shown). These effects on IAP levels were not caused by IAP degradation, as GST–Rpr failed to alter IAP levels when added to reticulocyte lysates, after translation had been blocked with cycloheximide (Fig. 5b).

Because the IAP constructs used in the reticulocyte lysates lacked native 5′-or 3′-UTR sequences, we considered it unlikely that the degradation-independent effects of Rpr were IAP-specific. Accordingly, when reticulocyte lysates were programmed with unrelated messages, the GST–Rpr protein also effectively dampened their expression (Fig. 5c). Again, this effect was not caused by protein degradation, as GST–Rpr addition did not affect levels of previously transcribed and translated proteins in reticulocyte lysates (Fig. 5c).

To assess the effects of Rpr on total protein synthesis, we added GST–Rpr to *Xenopus* egg extracts, which were translationally competent and transcriptionally inactive. These extracts were supplemented with ^{35}S-Met/Cys and high levels of zVAD-fmk to prevent caspase-mediated cleavage of translation factors. Addition of GST–Rpr or Rpr peptide to *Xenopus* egg extracts globally suppressed protein synthesis (Fig. 5d,e). Importantly, unrelated GST fusion proteins prepared in the same manner as GST–Rpr had no such effect (Fig. 5d,e). Rpr did not reduce protein levels by accelerating general protein degradation, as co-addition of GST–Rpr or Rpr peptide and cycloheximide to extracts after 45 min of translation did not result in destruction of nascent proteins (Fig. 5d,e). These data strongly suggest that the ability of Rpr to post-translationally destabilize proteins is specific to the IAPs. Thus, Rpr can decrease generalized translation in a manner distinct from its ability to accelerate the ubiquitin-mediated destruction of extant IAPs. Unlike the effect on IAP protein stability, the Rpr effect on translation did not require a free N terminus, as GST–Rpr was effective in translational inhibition. GST–Rpr$^{16-65}$, which lacks the first 15 amino acids of Rpr, also inhibited translation, confirming that the extreme N terminus of Rpr is dispensable for translational inhibition (Fig. 5e).

Although GST–Rpr was able to decrease IAP levels (Fig. 5b), the Rpr peptide was more effective in this regard. We hypothesized that the peptide might more effectively lower wild-type XIAP protein levels by simultaneously shortening XIAP half-life and inhibiting protein translation. We therefore returned to the reticulocyte lysate system to examine levels of the XIAP^{H467A}, as this mutant is not subject to Rpr-mediated degradation. When the XIAP mutant was examined in this system, we found that GST–Rpr indeed suppressed translation of this protein, as it had with other proteins tested. However, whereas the abundance of wild-type XIAP had been more dramatically reduced by the Rpr peptide than by GST–Rpr, the abundance of the XIAP ubiquitin ligase mutant was suppressed equally by both (compare Fig. 5b and f).

Despite the robust translational inhibition by Rpr *in vitro*, we wanted to determine whether we could detect such effects of Rpr in

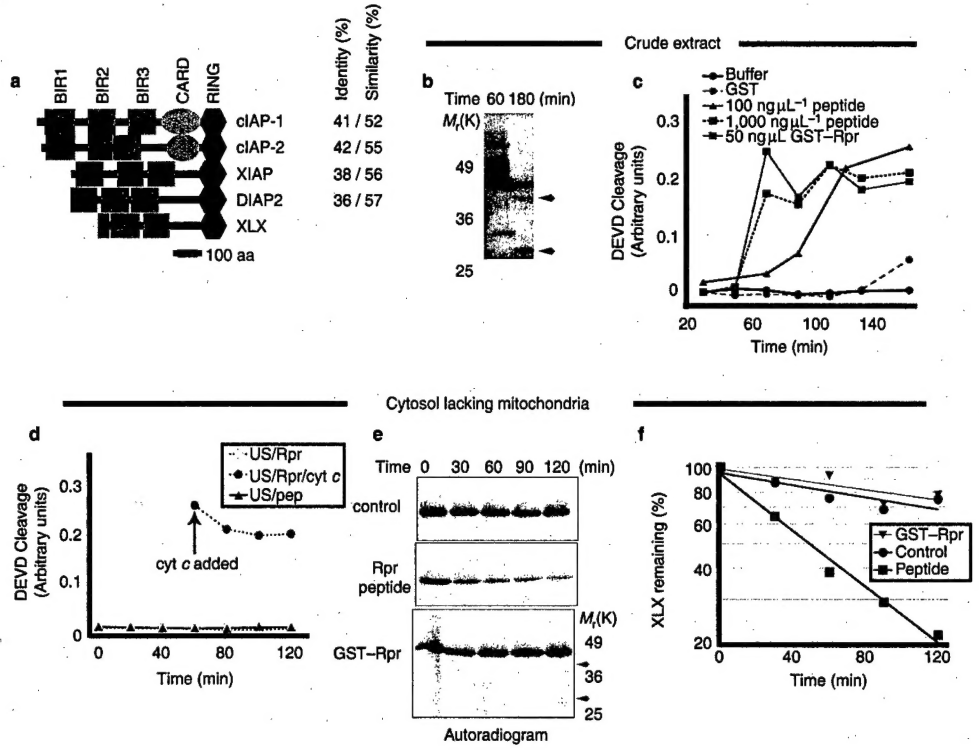

Figure 2 **XLX, a *X. laevis* XIAP homologue, is destabilized by Rpr peptide, but not by GST–Rpr. a**, A domain map drawn to the scale of XLX with three human IAPs: cIAP1, cIAP2, and XIAP, and *D. melanogaster* DIAP2 is shown. Grey boxes represent BIR domains, ovals represent caspase recruitment domains (CARD), and hexagons represent C-terminal RING domains. The percentage identity and similarity between residues of each protein to XLX are indicated. BLAST alignment of XLX to proteins in the non-redundant public database yielded chicken and human XIAP as the most similar clones. XLX has a similar domain structure to XIAP and DIAP2. **b**, GST–Rpr and radiolabelled XLX were added to crude *Xenopus* egg extract. Samples were resolved by SDS–PAGE and autoradiography at the indicated times. Arrowheads denote the ~40K and 30K XLX cleavage products. Molecular weight markers are shown for reference with **e** below. **c**, GST–Rpr, GST, Rpr peptide or peptide vehicle were added to crude *Xenopus* egg extract. Caspase activity was monitored by cleavage of the colorimetric peptide substrate DEVD–pNA. **d**, GST–Rpr (Rpr) or Rpr peptide (Pep) were added to *Xenopus* egg extract depleted of mitochondria by centrifugation (US). At 60 min, human cytochrome *c* was added to an aliquot of the extract containing GST–Rpr (arrow). Caspase activity was monitored as in **c** above. **e**, Rpr peptide, peptide vehicle (control) or GST–Rpr were added together with radiolabelled XLX into *Xenopus* egg extract depleted of mitochondria. Samples were taken at the indicated times and XLX protein levels were analysed by SDS–PAGE and autoradiography. Molecular weight markers are shown for comparison with **b** and arrows indicate the approximate position of expected XLX cleavage fragments (which are absent). Equal loading was verified by Coomassie blue staining of gels (data not shown). **f**, The autoradiogram in **e** was quantified using a phosphorimager.

intact cells. Accordingly, we injected whole *Xenopus* oocytes with zVAD-fmk and either *rpr* sense or anti-sense mRNA. After 12 h incubation to allow translation of the Rpr protein, we re-injected oocytes with ^{35}S-methionine, incubated them for a further 4 h, lysed the oocytes and assessed the level of total protein synthesis by measurement of TCA-precipitable radioactivity. The oocytes injected with zVAD-fmk and *rpr* sense mRNA incorporated approximately sevenfold less counts than the anti-sense controls (~1.1×10^5 cpm versus ~7.9×10^5 cpm). These data demonstrate that even when synthesized *de novo* within an intact cell, Rpr can inhibit protein translation. Consistent with these results, cotransfection of human cells with Rpr and GST reduced GST synthesis by ~30% in a pulse labelling experiment, despite the very low levels of Rpr produced in these cells (data not shown). Although these results were more modest than those obtained in reticulocyte lysates or oocytes, we have not been able to achieve comparable levels of Rpr in the intact tissue culture cells. However, even a moderate reduction in protein synthesis, coupled with a decrease in IAP stability, would synergize to produce an effective elimination of the IAPs.

In aggregate, our data suggest that Rpr eliminates IAPs by simultaneously stimulating their ubiquitin-mediated degradation and down-regulating total protein translation. This reduction in IAP levels by Rpr lowers the threshold for caspase function, thereby facilitating apoptotic progression.

Note added in proof: Several other papers in this issue also demonstrate that Reaper functions to stimulate IAP degradation[23–25]. *Additionally, another paper in this issue supports our findings that Reaper suppresses general protein translation*[26]. □

Methods

Cell culture, transfections, immunoblotting, and pulse-chase analysis

All cell culture reagents were obtained from Gibco (Rockville, MD) unless otherwise specified. HEK 293T cells were obtained from the American Type Culture Collection (ATCC) through the Duke Cell Culture Facility, and were maintained in MEM, which was supplemented with 10% foetal bovine serum, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids solution. The *Drosophila rpr* gene was cloned into pEBB using standard methods. For the immunoblots shown, 1×10^6 cells were plated in 100-mm dishes and transfected 24 h later using the Fugene 6 reagent (Roche Molecular, Indianapolis, IN) and 10 μg of total DNA, according the manufacturer's instructions. 24–48 h after

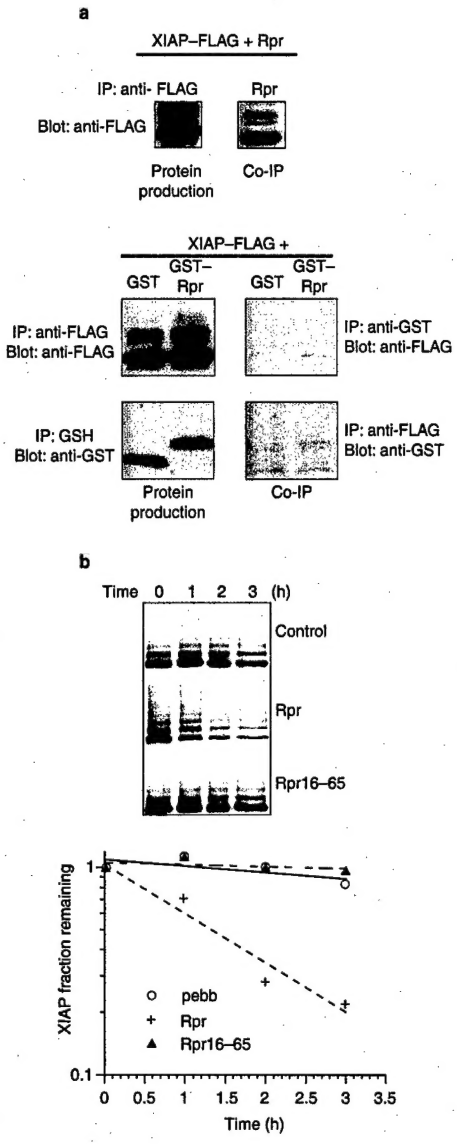

Figure 3. **An unblocked Rpr N terminus is required for IAP binding and stabilization. a**, HEK 293T cells were transfected with Rpr, GST alone or GST–Rpr and FLAG-tagged XIAP protein. Proteins were precipitated with anti-FLAG or anti-Rpr antibodies and blotted with anti-FLAG. Proteins were precipitated with glutathione–Sepharose or anti-FLAG, and detected by immunoblotting with anti-GST or anti-FLAG. Although GST–Rpr and XIAP failed to coprecipitate, the untagged Rpr control clearly coprecipitated with XIAP protein. **b**, A pulse-chase experiment was performed as in Fig. 1c, using either wild-type Rpr (Rpr) or untagged Rpr lacking its first 15 amino acids (Rpr^{16-65}). The results were then quantified.

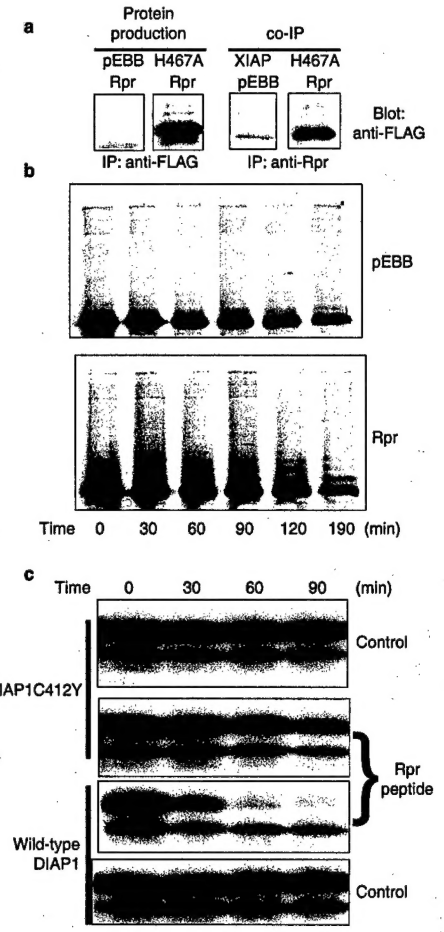

Figure 4. **Rpr does not destabilize the $XIAP^{H467A}$ ubiquitin ligase mutant. a**, HEK 293T cells were transfected with FLAG-tagged wild-type XIAP or FLAG-tagged catalytically inactive XIAP ($XIAP^{H467A}$) in conjunction with a control vector (pEBB) or untagged Rpr (Rpr). Samples were precipitated with an anti-FLAG antibody (left) or an anti-Rpr antibody (right), demonstrating a nearly quantitative association of the mutant XIAP with Rpr. **b**, HEK 293T cells were transfected with GST–$XIAP^{H467A}$ and either Rpr or vector control. Cells were then radiolabelled in a pulse-chase experiment, and the resulting radiolabelled GST–$XIAP^{H467A}$ proteins were analysed by autoradiography. **c**, Radiolabelled DIAP1 protein, either wild-type or the catalytically inactive $DIAP^{C412Y}$ mutant, was incubated in *Xenopus* egg extract lacking mitochondria, in the presence of Rpr peptide or vehicle control. The resulting radiolabelled protein levels were analysed by autoradiography.

transfection, cells were washed once in PBS, collected in lysis buffer (10 mM HEPES at pH 7.4, 50 mM potassium chloride, 2.5 mM magnesium chloride and 50 mM sucrose, plus 1× Complete protease inhibitor (Roche Molecular)) and briefly sonicated. Lysates were incubated for 10 min on ice and cleared by centrifugation at 10,000*g* for 10 min. Cleared lysates were then incubated with glutathione–Sepharose (Pharmacia) or the M2 anti-FLAG antibody (Sigma, St Louis, MO) and Protein G–agarose (Oncogene Research Products, Boston, MA) or K1 anti-Rpr antibody and Protein A–Sepharose (Sigma) at 4 °C for 1 h. The bead-bound material was washed three times in lysis buffer and released in 2×SDS sample buffer. This material was then separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes by standard methods. Membranes were blocked in PBS containing 0.1% Tween-20 and 5% dry milk. For immunoblotting to detect GST fusions, rabbit antiserum to GST was used at 1:3,000 in PBS containing 0.1% Tween-20 and 2% BSA, before incubation with Protein A–horseradish peroxidase (HRP; Amersham, Sunnyvale, CA) at 1:10,000. Immunoblots to detect FLAG-tagged proteins were handled similarly using the M2 anti-FLAG antibody (1 μg μl^{-1}) and goat anti-mouse-HRP (Jackson ImmunoResearch, West Grove, PA), whereas ubiquitin was detected using mouse anti-ubiquitin (1:100; Zymed, San Francisco, CA) and Protein A–HRP without pre-blocking the membrane. Blots were developed using Renaissance ECL reagents (NEN, Boston, MA) and exposed to Biomax ML film (Kodak, Rochester, NY). For pulse-chase analysis, 200,000 cells were plated per well in 6-well plates and transfected as above, except that a total of 1.5 μg DNA was used. 16–20 h after transfection, cells were washed once in prewarmed pulse medium (DMEM minus L-Met and L-Cys supplemented with 10% dialysed foetal bovine serum and 1 mM sodium pyruvate) and then incubated for 15 min in pulse medium to deplete Met and Cys levels. Cells

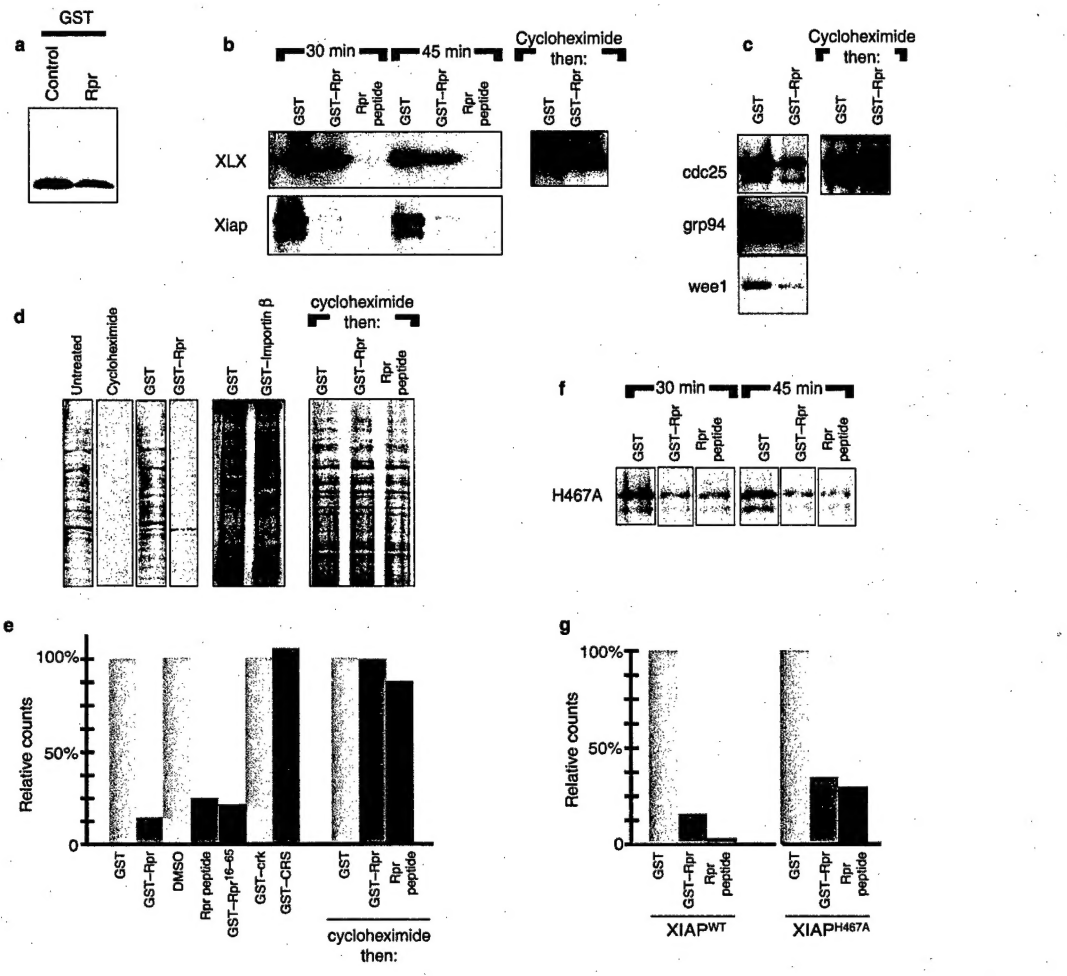

Figure 5 **Repression of translation by GST–Rpr and Rpr peptide. a**, HEK 293T cells were transfected with GST and either Rpr or vector control. Resulting GST protein levels were analysed by immunoblotting. **b**, XLX and human XIAP were added to rabbit reticulocyte lysates, which were supplemented with GST, GST–Rpr or Rpr peptide. Aliquots were collected at 30 and 45 min, and resolved by SDS–PAGE (left). Rabbit reticulocyte lysates were programmed with XLX and allowed to transcribe and translate for 45 min. Translation was stopped with the addition of cycloheximide and the lysates were incubated for an additional hour with GST or GST–Rpr before resolution by SDS–PAGE (right). **c**, Cdc25, Grp94 and Wee1 cDNAs were added to reticulocyte lysates and supplemented with GST or GST–Rpr, as above (left). The rabbit reticulocyte lysates were also allowed to transcribe and translate for 45 min, after which translation was stopped with cycloheximide (right). Samples were then incubated for an additional hour with GST or GST–Rpr, as above. **d**, Translationally competent, but transcriptionally inactive, *Xenopus* egg extracts were supplemented with ^{35}S-Met/Cys and zVAD-fmk plus egg lysis buffer (Untreated), cycloheximide, GST, GST–Rpr, or GST–importin-β as a non-specific control (left). Translation was allowed to proceed for 45 min and products were resolved by SDS–PAGE. Egg extracts supplemented with ^{35}S-Met/Cys and zVAD-fmk were allowed to translate for 45 min, translation was stopped with cycloheximide and extracts were incubated for an additional hour with GST, GST–Rpr or Rpr peptide (right). **e**, Samples prepared as in **d** were subjected to TCA precipitation and quantified by scintillation counting. Additionally, control proteins (GST–cyclin B1, GST–CRS, GST–Crk), peptide vehicle (DMSO), GST–Rpr$^{16–65}$ or Rpr peptide were assayed in the same manner. The resulting incorporated counts were TCA precipitated and scintillation counting was performed as above. **f**, XIAP^{H467A}, a mutant unable to function as a ubiquitin ligase, was added to rabbit reticulocyte lysate, which was supplemented with GST, GST–Rpr or Rpr peptide, and aliquots were collected at 30 and 45 min before resolution by SDS–PAGE. **g**, Phosphorimager quantification of wild-type XIAP and XIAP^{H467A} protein levels from **b** and **f**.

were then radiolabelled for 30 min with pulse medium containing 200 μCi ml^{-1} of ^{35}S-Trans label (ICN, Costa Mesa, CA). After labelling, cells were washed once with their normal culture medium and incubated in the complete medium for the chase times indicated. Radiolabelled proteins were harvested by rinsing the cells once in PBS and then lysing in 0.1% NP40, 150 mM sodium chloride, 50 mM HEPES at pH 7.4 and 1 mM EDTA, plus 1× protease inhibitors as above. Cell lysates were cleared by incubation on ice and centrifugation as above. GST–fusion proteins were captured on GSH–Sepharose and separated by SDS–PAGE as above. Gels were soaked in 1 M salicylate (Sigma) for 30 min before drying and overnight exposure to Biomax MR film (Kodak).

Cloning of XLX

A probe derived from the RING domain of human cIAP1 was generated using the Random Primed Labelling kit (Roche Molecular) and used to screen ~500,000 clones of a λ-zap *Xenopus* gastrula library at low stringency. Several clones >1 kB were isolated, excised and partially sequenced. A secondary screen was performed for one of the clones isolated using oligonucleotides designed to anneal to the linker region between the BIR and RING domains. The probe was generated by PCR with radiolabelled nucleotides (oligonucleotides: 5′-GATCTTTAGAAGCCCAGAGTCCTCTCCT-3′ and 5′-GATCCTTGCTCTGAATTAGACTTGCCAC-3′). This screen failed to isolate any larger clones. The

~1.6 kB cDNA was fully sequenced and deposited in GenBank. A BLAST alignment was performed using both the complete cDNA and the longest uninterrupted open reading frame. Domain analysis was performed using InterPro (http://www.ebi.ac.uk/interpro/).

Extract preparation

Preparation of crude interphase egg extracts (CS) was performed as previously described[21]. To fractionate the crude egg extract into cytosolic (US) and membranous components, the crude extract was centrifuged further at 200,000*g* for 1 h in a Beckman TLS-55 rotor using a TL-100 centrifuge. The cytosolic fraction (ultra-S or US) was removed and recentrifuged for an additional 25 min at 200,000*g*. These reconstituted extracts were supplemented with an energy regenerating system consisting of 2 mM ATP, 5 mg ml^{-1} creatine kinase, and 20 mM phosphocreatine (final concentrations).

Production of GST, GST–Rpr and Rpr peptide

GST and GST–Rpr were prepared as previously described[21]. Rpr was also generated as a full-length, untagged synthetic peptide by B. Kaplan (City of Hope, Beckman Research Institute). The peptide was received as a lyophilized powder, which was stored solid at 4 °C. Before use, the peptide was resuspended in dimethylsulphoxide (DMSO) at 10 mg ml^{-1}, and then diluted to 1 mg ml^{-1} in egg lysis buffer (10 mM HEPES at pH 7.4, 50 mM potassium chloride, 2.5 mM magnesium chloride, 50 mM sucrose and 1 mM dithiothreitol (DTT)).

DEVD assay

Recombinant GST, GST–Rpr, Rpr peptide or peptide vehicle (10% v/v DMSO in egg lysis buffer) was added at a 1:10 dilution to CS or US extract containing energy regenerating mix (see above). At the indicated times, 3-μl aliquots were withdrawn and incubated with 90 μl of assay buffer (50 mM HEPES at pH 7.5, 100 mM sodium chloride, 0.1% CHAPS, 10 mM DTT, 1mM EDTA and 10% glycerol) containing 200 μM Ac–DEVD pNA colorimetric substrate (BioMol, Plymouth Meeting, PA). Caspase-3 activity was monitored by the measurement of absorbance at 405 nm using a LabSystems MultiSkan MS microtiter 96-well plate reader (Helsinki, Finland).

In vitro translation

XIAP ORFs were subcloned using standard techniques into pBS II-SK and pSP64T, a TNT expression vector with flanking 5′ and 3′ β-globin UTR and a polyadenosine tail. To produce radioactive protein for half-life assays, Cdc 25, Grp94, Wee1, XLX, wild-type XIAP/DIAP, and $XIAP^{H467A}$/$DIAP^{C412Y}$ templates were added at 20 ng μl^{-1} to rabbit reticulocyte lysate (Stratagene, La Jolla, CA) containing 1 μCi μl^{-1} of Trans label, 1× (minus-Cys, minus-Met) amino acid mix and other components, in accordance with the manufacturer's protocol. For *Xenopus* stability assays, the reaction was stopped after 90 min and proteins were snap frozen in liquid nitrogen for later use. For translation inhibition assays, reticulocyte lysate reactions were supplemented with 100 ng μl^{-1} of recombinant GST or GST–Rpr proteins, or Rpr peptide. Aliquots were withdrawn at the indicated times, resolved by SDS–PAGE, quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA), and exposed to film. Protein degradation was assayed by allowing translation to proceed for 45 min, at which point cycloheximide was added to a final concentration of 500 ng μl^{-1}. Subsequently, GST–Rpr, Rpr peptide or GST were added to a final concentration of 100 ng μl^{-1} and the mixture was incubated for 60 min at room temperature. Translated proteins were resolved by SDS–PAGE and quantified with the phosphorimager as described above.

Xenopus extract stability assay

In vitro-translated proteins were added on ice at 1:10 dilution into 100 μl of either CS or US lysate (with energy mix) that had been supplemented with 100 ng μl^{-1} GST, GST–Rpr, or Rpr peptide. Where indicated, zVAD-fmk (BioMol) or DMSO vehicle was also added at a final concentration of 100 μM (data not shown). Samples were shifted to room temperature or 4 °C and 20-μl aliquots were withdrawn at the indicated times, mixed with 40 μl SDS loading buffer and flash frozen in liquid nitrogen. Samples were thawed by boiling for 5 min and then assayed by SDS–PAGE before quantification on a phosphorimager and exposure to film.

Xenopus extract translation assay

In vitro translation assays using *Xenopus* extract were conducted by adding 1 μCi μl^{-1} of Trans label, 100 μM zVAD-fmk and 100 ng μl^{-1} of recombinant GST, GST–crk, GST–CRS (Cyclin B cytoplasmic retention sequence), GST–importin-β, GST–Rpr, GST–Rpr^{16-65} proteins or Rpr peptide to crude egg extract. The extent of protein translation was assayed by SDS–PAGE analysis and quantified by autoradiogram and phosphorimager, or by TCA precipitation (80 μg of extract in 20% TCA). Rpr-induced degradation was assayed as in reticulocyte lysates (above), save that in addition, total translated protein was also quantified by TCA precipitation as described.

Oocyte micro-injection and translation assay

Stage VI oocytes of *X. laevis* were prepared for micro-injection as described[22]. 25 nl of 0.4 μg μl^{-1} sense or antisense *rpr* RNA produced using the mCAP RNA capping kit (Stratagene) were injected into oocytes along with 100 μM zVAD-fmk. *Rpr* expression was allowed to proceed overnight, before an injection of Trans Label (25 nl of 10 μCi μl^{-1}). 25 oocytes injected with *rpr* sense or antisense RNA were collected 4 and 5 h after Trans label injection. The oocytes were lysed in buffer (5 mM HEPES at pH 7.8, 88 mM sodium chloride, 1 mM potassium chloride, 1 mM magnesium sulphate, 2.5 mM $NaHCO_3$, 0.7 mM calcium chloride and 50 ng μl^{-1} apropotein/leupetin/ cytochalasin B) by centrifugation at 16,000*g* for 15 min. Total protein translation was assayed by TCA precipitation (80 μg of oocyte extract in 20% TCA) as described.

Accession numbers

X. laevis XIAP (XLX) was submitted to GenBank and given the accession number AF468029.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.